RAPID DETECTION OF DRUG RESISTANT TUBERCLE BACILLI BY SLIDE CULTURES OF SPUTUM*

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WITH the widespread use of antimicrobial therapy in tuberculosis, there is urgent need for early detection of the emergence of drug resistant strains of tubercle bacilli. The methods most generally employed for the determination of susceptibility of Mycobacteria to antimicrobial agents require long periods of time—often as long as two months. For this reason in many instances, as Dr. Amberson has already stated, the physician finds it necessary to change drug therapy before laboratory results are available.

Slide culture techniques have shortened appreciably the time required for the detection of growth of tubercle bacilli. A paper by Reed and Morgante[1] summarizes their own investigations and provides an extensive review of the literature dealing with such methods. These techniques are being used for drug susceptibility determinations in foreign countries, but have received relatively little attention in the United States.

I should like to discuss some results obtained from slide cultures of tuberculous sputum and to emphasize in particular the advantage of this method for the early detection of drug resistant organisms.

The procedure being used at present in our laboratory is a slight modification of that previously reported[2].

For the protection of personnel all manipulations with the exception of the homogenization of infectious material are carried out in a hood such as illustrated in Figure 1. This is kept under negative pressure and is equipped with ultraviolet light to provide sterilization of the interior chamber before and after slide cultures are made.

Sputum specimens are collected over a 24 hour period. In patients

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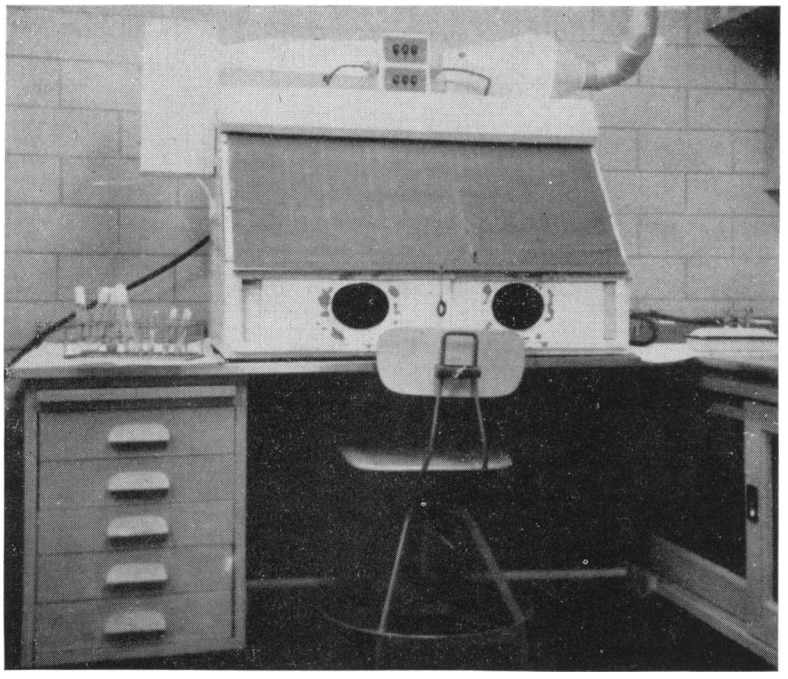


Fig. 1—Safety cabinet*. **

* Designed at the Rockefeller Institute for Medical Research.
** Shade pulled over glass surface when ultraviolet lights are on.

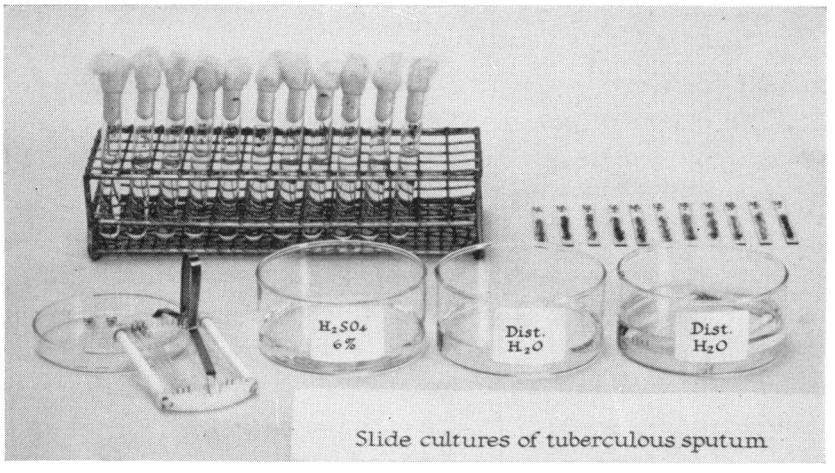


Fig. 2—Steps in the preparation of slide cultures.

receiving drug therapy all antimicrobial agents are withheld for a period of 24 hours preceding the sputum collection as well as during the 24 hour collection period. Homogenization of the specimen is accomplished by shaking the original container in a paint shaker for one to three minutes. The different steps involved in the preparation of the cultures are illustrated in Figure 2. Smears of the homogenate are made on a series of small glass slides—the number being determined by the different antimicrobial agents under test. These slides are merely the standard microscopic slides cut longitudinally into three strips.* The smears are left to dry in Petri dishes at room temperature after which they are placed in a teflon rack** which can hold a maximum of 16 slides. (Teflon has the advantage of being a plastic easily tooled, is inert in strong chemical solutions, and can withstand autoclaving.) The filled rack is then immersed in 6 per cent sulfuric acid and left for six minutes —a time adequate to destroy contaminating microorganisms other than tubercle bacilli in the sputum. The acid is removed by two separate rinses in sterile distilled water contained in crystallizing dishes. The slides are subsequently removed from the racks by means of forceps and deposited without drying into Wassermann tubes—one containing the control medium only (Dubos basal liquid medium plus oleic acid albumin), the remainder of the series containing five-fold dilutions of the drugs to be tested. The tubes are incubated at 37° C. in an upright position for five or six days after which the slides are removed and allowed to dry at room temperature. After fixation with absolute methyl alcohol, they are stained by an acid-fast method using pararosanilin instead of basic fuchsin. The staining with carbol-pararosanilin is carried out for ten minutes on a metal staining rack to insure killing of the viable organisms. The slides are then ready for microscopic evaluation.

Figure 3 presents the gross appearance of a set of slide cultures in which the tubercle bacilli are susceptible to isoniazid and streptomycin. It is obvious that marked multiplication of tubercle bacilli has taken place on the control slide C—i.e., the one grown in medium containing no drug. No multiplication has taken place on the slides grown in the presence of 1, 5, or 25 micrograms per milliliter of isoniazid or similar concentrations of streptomycin. Examination of these six slides under oil immersion reveals the presence of bacilli which were in the original

* Obtained commercially from Scientific Glass Apparatus Co., Inc., 100 Lakewood Terrace, Bloomfield, New Jersey.

**Designed by Mr. Nils A. Jernberg, Instrument Shop, Rockefeller Institute for Medical Research.

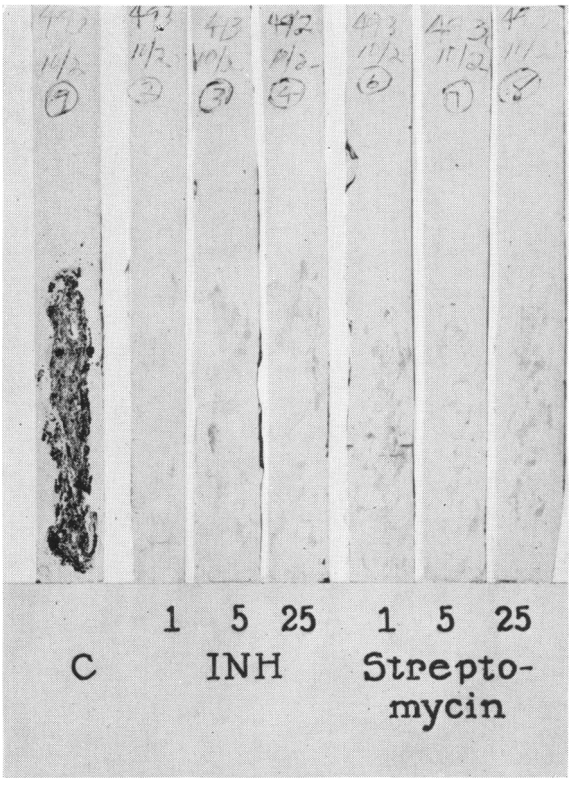


Fig. 3—Slide cultures of tubercle bacilli susceptible to isoniazid and streptomycin*.

C=control medium without drug.

1, 5, 25=final concentration (microgram per ml. of medium) of indicated drug.

*=Standard microscopic glass slides cut by hand before commercial product became available.

smear occurring singly or in small groups of four to five organisms. In contrast, examination of the control slide shows extensive multiplication with the typical morphology characteristic of virulent tubercle bacilli. This can be seen in the photomicrographs, Figures 4a and 4b. Tubercle bacilli arranged in parallel and forming the typical long, winding, cord-like pattern are seen in the control slide culture (Figure 4a). No multiplication of organisms is observed on slide cultures grown in the presence of isoniazid or streptomycin which are represented in Figure 4b.

Not all sputum specimens are as heavily positive for acid-fast bacilli

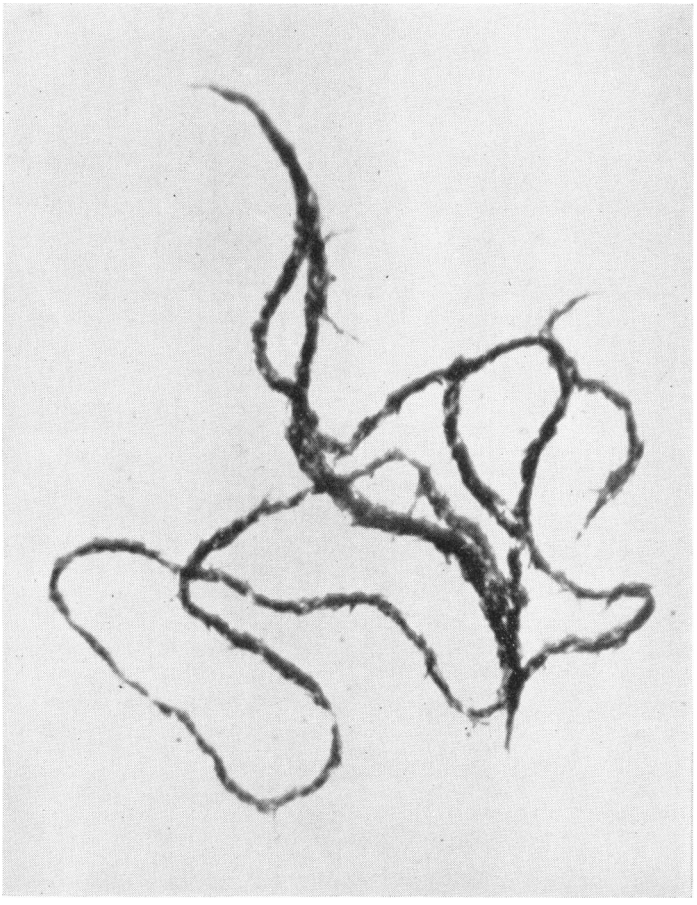


Fig. 4a—(Magnification 1315 X)

Multiplication of tubercle bacilli in control medium without drug.

as the one shown in Figure 3. The range which may be expected is illustrated in the photomicrograph (Figure 5). These are typical areas at low power magnification (124x) of slide cultures from sputa of three patients. It can readily be seen, however, that even when relatively few organisms are present in the inoculum (Figure 5-c), multiplication can be detected within seven days.

The slide culture technique for testing drug resistance is generally applicable only to specimens in which bacilli are demonstrable by direct smear. Tables I and II serve to demonstrate this point.

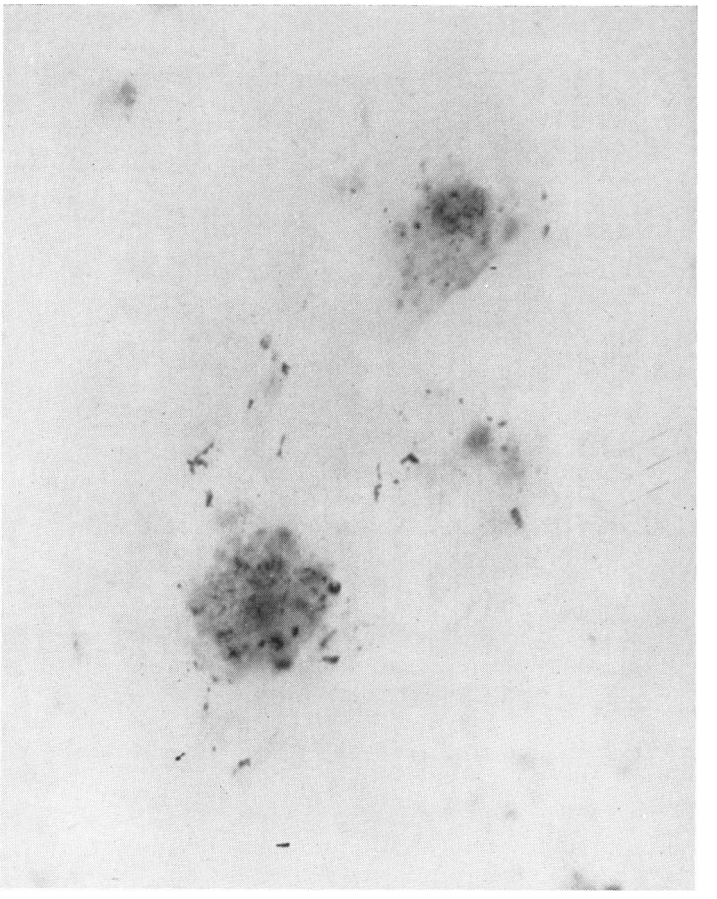
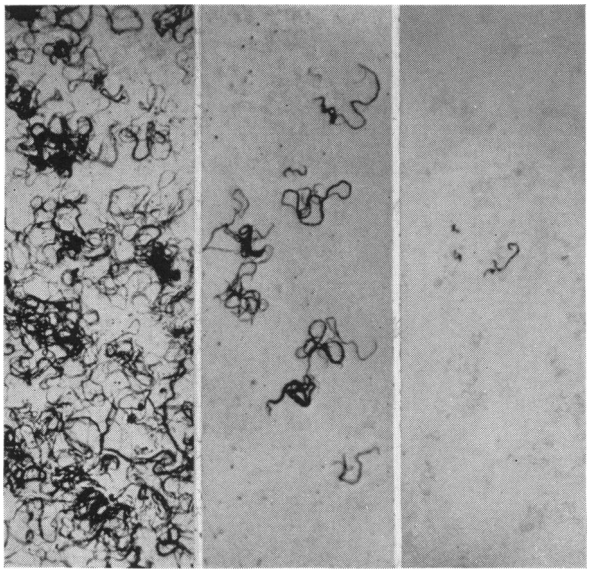


Fig. 4b—Appearance of tubercle bacilli present in original smear but non-multiplying in the presence of isoniazid or streptomycin.

Table I presents results obtained from a series of sputum specimens which were positive for acid-fast bacilli by direct smear. Three specimens were obtained from each patient at intervals of two weeks. After the sputum was smeared for slide culture, it was then concentrated in a routine manner by NaOH digestion, centrifugation and neutralization with HCl. The sediment obtained was washed and recentrifuged before inoculation onto egg slants (Löwenstein-Jensen medium). All slide cultures were examined after an incubation period of five days and all showed multiplication of acid-fast bacilli (microcolonies of tubercle bacilli could have been detected as early as three days). The figures in



a. b. c.

Fig. 5*—(Magnification 124 X)
Positive slide cultures of sputa from three different patients.

the column on the right indicate the incubation time required for the appearance of visible colonies of tubercle bacilli on egg slants. Not only is a much longer period required—even up to 42 days in the case of L. S. —but there is great variability among sputum specimens from the same patient in all instances. This was not observed when slide cultures were used.

Table II presents comparative results obtained by slide culture and egg slant inoculation when the sputum specimens were negative for acid-fast bacilli by direct smear. It can readily be seen that all the slide cultures were negative. One must bear in mind, however, that the inoculum deposited on the egg slants was approximately 20 times that used for slide cultures—therefore, it is not surprising that the slide cultures are negative when only a very few colonies of tubercle bacilli (less than 10) are recovered from sediments of sputum. (Sediment obtained after NaOH digestion of sputum does not adhere to slides during the process of slide culturing.)

* Previously published in *American Review of Tuberculosis and Pulmonary Diseases* ref. 2. Reprinted by permission.

TABLE I—GROWTH OF TUBERCLE BACILLI FROM SPUTA*
(POSITIVE BY SMEAR)

Patient	*Time (in days) required for detection of growth*	
	Slide culture	*Egg slant***
JM	5	19
	5	30
	5	40
CT	5	25
	5	12
	5	17
JMa	5	12
	5	18
	5	18
LS	5	24
	5	42
	5	21
MV	5	21
	5	18
	5	21

* Condensation of results previously published[2].
** More than 100 colonies or confluent growth on all slants.

TABLE II—GROWTH OF TUBERCLE BACILLI FROM SPUTA*
(NEGATIVE BY SMEAR)

Patient	*Slide Culture*		*Egg Slant (inoculated in triplicate)*	
	Day of observation	*Growth*	*Day of detected growth*	*No. of colonies per slant*
GG	8	Neg.	17	2, 3, 5
	5	"	33	1, 1, 0
	5	"	41	1, 0, 1
MB	5	"	58	1, 0, 0
	5	"	50	9, 7, 0
JM	5	"	21	1, 0, 0
ET	5	"	23	8, 3, 5
BB	5	"	25	10, 2, 4

* Results previously published[2].

TABLE III—DRUG RESISTANCE OF TUBERCLE BACILLI FROM SPUTA*

Patient	*Time (in days) required to assess resistance*													
	Slide Culture									*Egg Slant*				
	Control no drug	*Isoniazid micrograms/ml.*				*Streptomycin micrograms/ml.*				*Control no drug*	*Isoniazid micro-grams/ml.*		*Streptomycin micro-grams/ml.*	
	0	1	5	25	125	1	5	25	125	0	1	5	10	100
JL	5	—**	—	—	—	—	—	—	—	19	—	—	—	—
MB	5	5	5	5	—	—	—	—	—	19	19	19	—	—
BN	6	6	6	6	—	—	—	—	—	11	30	30	—	—
MV	5	—	—	—	—	5	5	5	—	18	—	—	23	23
CT	5	—	—	—	—	5	5	—	—	17	—	—	38	—
CT	5	—	5	—	—	5	5	—	—	12	40	—	53	—
LS	7	7	7	7	—	7	7	7	7	46	46	46	46	46

* Condensation of results previously published[2].
**—Negative (no multiplication).

A comparison of the two methods for the determination of resistance of tubercle bacilli to isoniazid or streptomycin is illustrated in Table III. Four concentrations of isoniazid or streptomycin were used in the medium for slide cultures, and the two standard concentrations of isoniazid or streptomycin in the egg medium. This chart presents results of slide cultures stained at five, six or seven days of incubation. Later readings of duplicate sets stained after two weeks' incubation gave identical results as far as resistance to the antimicrobial agents is concerned. It is immediately evident that earlier detection of growth of tubercle bacilli was obtained from all specimens by the slide culture technique as compared with egg slants.

Sputum of the first patient, J. L., yielded acid-fast organisms which were sensitive to concentrations of isoniazid or streptomycin as low as 1 microgram/ml. In contrast, the organisms from M. B. were resistant to 25 micrograms of isoniazid but sensitive to streptomycin. In the case of B. N., who also had organisms resistant to 25 micrograms of isoniazid, this fact was not obtained by the egg slant method until almost three weeks after the control culture had grown, 11 days vs. 30—whereas by slide culture the organisms grew in the presence of isoniazid at the same rate as the control. The organisms from M. V. were resistant to strepto-

mycin and again the lag in determination of resistance can be noted with the egg slants. The same holds true for the first specimen from C. T. With a specimen obtained from C. T. two weeks later, the development of resistance to isoniazid as well as to streptomycin is noted by slide cultures within five days but with egg slants resistance to isoniazid was not apparent until 40 days of incubation, resistance to streptomycin not until 53 days. The last patient, L. S., had organisms highly resistant to both isoniazid and streptomycin—the endpoint of streptomycin resistance not being obtained. Moreover, a positive culture was not detected on the control egg slant or drug tubes until 46 days. This is in striking contrast to the results obtained within seven days by slide cultures.

Discussion and Summary

The over-all results in terms of resistance or susceptibility of tubercle bacilli to various concentrations of isoniazid or streptomycin were essentially the same with either the slide culture method or the egg slant inoculation. However, the difference in time required for the determination is quite striking—one week or less with slide cultures as contrasted with three to six weeks with egg slants. It should be re-emphasized also that with slide cultures resistant organisms were demonstrable as soon as growth was determined on the control slide, whereas in many instances there was a great delay in the appearance of resistant colonies on egg slants even after the control tube was positive.

Slide cultures of tuberculous sputum have the following advantages over the routine method of solid media inoculation:

1. Foremost, the resistance of tubercle bacilli to antimicrobial agents can be detected at an early date,
2. The time-consuming procedure of digesting, centrifuging, neutralizing and washing of the specimen can be eliminated, and
3. The actual degree of resistance is perhaps more accurate, since inspissation of medium and prolonged incubation are avoided, both of which may lead to destruction of antimicrobial agents.

The fact that the method is applicable only to those sputum specimens containing tubercle bacilli demonstrable by direct smear is not so severe a limitation as it may seem upon first thought. It is precisely in those instances when patients are excreting large numbers of organisms that an early report of resistance is of most value, i.e., at the start of

therapy in a newly diagnosed case, or when the course is unfavorable. Rapid detection of resistance may become much more important in the future if primary infection with drug-resistant strains is more frequently encountered.

All photographs, with the exception of Figure 1, were taken by Mr. Julian Carlile, Rockefeller Institute for Medical Research.

REFERENCES

1. Reed, R. W. and Morgante, O. Recent advances in the laboratory diagnosis of tuberculosis, *Amer. J. med. Sci. 231:* 320-37, 1956.
2. Pierce, C. H., Hirsch, J. G. and Schaedler, R. W. Rapid detection of drug-resistant tubercle bacilli in sputum by slide cultures, *Amer. Rev. Tuberc. Pulmonary Dis. 75:*331-37, 1957.